PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : A01H 4/00

A1

(11) International Publication Number: **WO 00/16610**

(43) International Publication Date: 30 March 2000 (30.03.00)

(21) International Application Number: PCT/SI99/00022

(22) International Filing Date: 22 September 1999 (22.09.99)

(30) Priority Data:
P-9800247 24 September 1998 (24.09.98) SI

(71) Applicant *(for all designated States except US)*: UNIVERZA V LJUBLJANI, BIOTEHNIŠKA FAKULTETA [SI/SI]; Jamnikarjeva 101, 1000 Ljubljana (SI).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: BOHANEC, Borut [SI/SI]; Ježa 76b, 1231 Ljubljana–Črnuče (SI). LUTHAR, Zlata [SI/SI]; Sebeborci 63, 9221 Martjanci (SI).

(74) Agent: ITEM, D.O.O.; Resljeva 16, 1000 Ljubljana (SI).

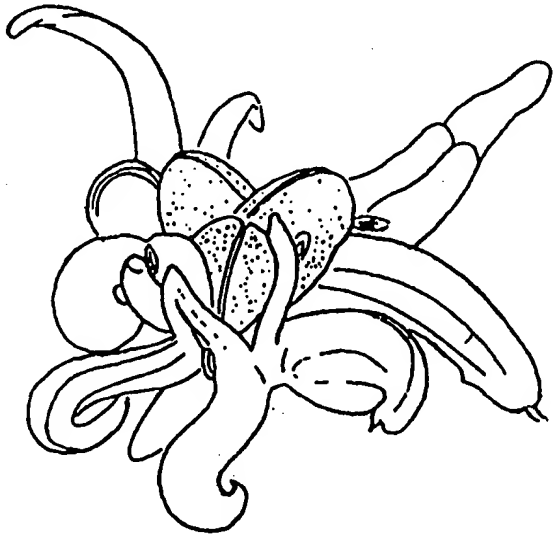
(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: A PROCESS FOR THE INDUCTION OF DIRECT *IN VITRO* ORGANOGENESIS IN ONION

(57) Abstract

A process is described leading to the formation of direct *in vitro* organogenesis omitting the callus stage in onion, initiated from generative organs, particularly flowers or ovaries. Embryogenic tissues, formed in the process of regeneration are suitable for further attempts at genetic transformations. Direct somatic organogenesis is a method that provides a high degree of clonal propagation (micropropagation) starting from individual donor plants.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

A process for the induction of direct *in vitro* organogenesis in onion

Field of invention

This invention refers to a field of plant biotechnology, specifically to a new process for the induction of direct *in vitro* organogenesis in onion, with a specific application use in micropropagation or genetic transformation in onion.

Onion (*Allium cepa* L.) is the second most important vegetable species worldwide and is produced in almost all climatic regions. It can be multiplied by seeds, sets or vegetatively. Vegetative *in vitro* methods are used for multiplication of valuable breeding lines including maintaining male sterile lines, used in hybrid seed production. Methods of direct *in vitro* organogenesis can be further used for successful genetic transformation in onion.

Prior Art

Publications concerning *in vitro* protocols in onion report of axillary and adventitious shoot proliferation of onion.

Micropropagation of onion presents several difficulties. Two different tissues have mainly been used for induction of shoot cultures - the first being inoculation of scale bases excised from the basal parts of bulbs or onion sets. This approach was taken in the following studies:

Hussey, G. (1978) *In vitro* propagation of the onion *Allium cepa* L. by axillary and adventitious shoot proliferation. Sci Hortic 9: 227-236;

Fujieda K., Matsuoka N., Fujita Y. (1979). Vegetative multiplication of onion (*Allium cepa* L.), through tissue culture. J Japan Soc Hort Sci 48: 186-194;

Hussey G., Falavigna A., (1980) Origin and production of *in vitro* adventitious shoots in the onion (*Allium cepa* L.). J. Exp. Bot. 31: 1675-1686;

Kahane R., Rancollac M., Teyssendier de la Serve B. (1992) Long-term multiplication of onion (*Allium cepa* L.) by cyclic shoot regeneration *in vitro*. Plant Cell Tiss Org Cult 28: 281-288.

Another approach used immature flower buds as a starting tissue. Matsubara S., Hihara H. (1978) Onion bulblet regeneration on receptacles *in vivo* and *in situ*. J Japan Soc Hort Sci 46: 479-486 reports on the use of bases of immature inflorescences.

Pike LM., Yoo KS. (1990) A tissue culture technique for clonal propagation of onion using immature flower buds. Sci Hortic 45: 31-36 and Mohamed-Yassen Y., Splittstoesser WE., Litz RE. (1993) *In vitro* bulb formation and plant recovery from onion inflorescences. Hort Science 28: 1052 used parts of immature inflorescences or individual immature flowers as explants.

Callus tissues have been induced on a wider range of explant tissues, including bulb, set or seedling radicle (Dunstan DI. and Short KC. (1978) Shoot production from onion callus cultures. Sci. Hortic. 9: 99-110. Attempts have been made to induce embryogenic callus from seedling leaf sheets, immature sexual embryos, immature unfertilized ovules, mature basal plates (Phillips CG, Luteyn KJ (1983) Effects of picloram and other auxins on onion tissue cultures. J Amer Soc Hort Sci 108: 948-953).
The most frequently studied plant growth regulators (phytohormones) for shoot induction have been naphtaleneacetic acid (NAA) and 6-benzylaminopurine (BAP), while for callus induction, picloram has been shown to be superior to NAA or 2,4-dichlorophenoxyacetic acid (2,4-D).

A characteristic of published protocols was a relatively low number of shoot formations per donor plant, protocols based on bulb scale parts resulting in up to 10 shoots per explant, while the explant number per one bulb was limited (Fujieda et al. 1979). Protocols based on immature flower buds resulted in about 10% induced flowers, which produced about 5 shoots per flower (Pike and Yoo 1990). Mohamed-Yassen et al. 1993 spliced immature inflorescences into 4 parts and achieved 10.6 shoots per explant making 42.4 shoots per inflorescence. None of the cited articles described a method of direct shoot organogenesis, which is the subject of this invention.

In vitro grown shoots can be subcultured according to Kahane et al. 1992, which is briefly based on swelling basal shoot parts, followed by splitting and further re-growth of micropropagated shoots. This multiplication cycle requires 3-4 months. On the other hand, the protocol published for somatic embryogenesis (Phillips and Luteyn 1983) required the induction of callus induced from meristem tips, seedling roots, mature embryos, immature fertilized ovules and parts of mature basal plates.

Description of Invention with Working Examples

It is evident from the prior art references that an efficient method of direct *in vitro* organogenesis in onion, in which multiple shoot structures are induced on mature flowers or ovaries excluding the callus stage, efficient on majority of tested cultivars, has not yet been developed. Such a method is advantageous for two reasons. Primary the formation of multiple shoot structures resulting in efficient vegetative multiplication of plants started from single individual plants. Secondary the use of direct somatic organogenesis as an important step in genetic transformation studies using a biolistic approach or *via* the *Agrobacterium* infection and similar methods.

We have now surprisingly found hat mature onion flowers or ovaries could be induced to form direct somatic organogenesis leading to the formation of multiple organogenic structures, which in later development form multiple shoots. The induction process and components of the media are very similar to those used for gynogenic embryo induction in onion (Bohanec B., Jakše M., Ihan A., Javornik B. (1995) Studies of gynogenesis in onion (*Allium cepa* L.): induction procedures and genetic analysis of regenerants. Plant Science 104: 215-224; Jakše M., Bohanec B., Ihan A.(1996). Effect of media components on the gynogenic regeneration of onion (*Allium cepa* L.) cultivars and analysis of regenerants. Plant Cell Rep 15: 934-938. The major differences between the protocol used for gynogenesis and the claimed process (protocol 2) are the use of BDS medium (Dunstan DI., Short KC. (1977) Improved growth of tissue cultures of onion (*Allium cepa* L.). Physiol Plant 41: 70-72) instead of B5 induction medium (Gamborg OL., Miller RA., Ojima K. (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50: 151-158), elevated inositol and vitamin contents, the use of gellan-gum instead of agar solidified media and a shorter induction treatment.

These relatively small differences cause a completely different regeneration response of the same onion organs. We believe that such responses have not been previously described.

The claimed invention is a process for induction of direct *in vitro* organogenesis in onion, comprising the steps of:

(i) Inoculation of flower buds at a mature stage but before dehiscence on induction media, which contain adequate concentrations of phytohormones, growth regulators and gelling agents for initiation of direct somatic organogenesis. Growth regulators at this stage include effective mixture of auxins and cytokinins, other media components include macro and micro elements, vitamins, inositol, proline, carbohydrates and gelling agents.

(ii) After an appropriate time of induction, transfer of explants from induction to differentiation media which contain adequate concentrations of cytokinin, macro and micro elements, vitamins, inositol, proline, sucrose and gelling agents, and growth until the occurrence of direct organogenesis.

(iii) Optional removal of perianth before the transfer from induction to differentiation media and culture of extracted ovaries. The following steps are the same as described in (i) and (ii) leading to induction of direct somatic organogenesis.

(iv) Separation of globular embryogenic structures developed as described in (ii) and (iii) followed by transfer of elongated shoots to media for root development or maintenance of compact organogenic structures.

(v) Acclimatization of previously (iv) mentioned rooted shoots.

Characteristic features of the claimed invention are described in the following paragraphs, however the scope of invention is not limited therewith. The invention includes all possible variations which are obvious to the person skilled in the art.

The applied differentiation and induction media contain as solidifiers gellan-gum, mixture of gellan-gum and agar, or only agar.

The duration of the growth on induction media is from 3 to 12 days.

The induction and differentiation media contain sucrose, glucose or maltose as a source of carbohydrates.

The induction and/or differentiation media contain 25-100 g/l of sucrose.

In addition to cytokinin the induction media contain 2,4-dichlorophenoxyacetic acid or picloram as a sources of auxins.

The induction media contain auxin or auxin and 6-benzylaminopurine, thidiazuron or isopentenyladenin (2ip) as sources of cytokinins.

The differentiation media contain thidiazuron or 6-benzylaminopurine as sources of cytokinins.

The induction and differentiation steps are performed in light or in darkness.

The composition of macro and micro elements in induction and differentiation media corresponds to BDS media prepared according to Dunstan and Short, 1977, B5 media (Gamborg et al. 1968) or MS media (Murashige and Skoog, 1962*).

*Murashige T., Skoog F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Detailed Description of the Process Embodiments

The plant material used in the following Examples originated from different, publicly available sources, cultivars were received from genebanks or were purchased at retail, and inbred lines were received from the US public breeding program (Dr. M.J. Havey, USDA, Madison, Wisconsin, USA).

Various genotypes of onion were used in these experiments: Belokranjka (Slovenia), Ptujska rdeča (Slovenia), Stuttgarter Riesen, Timor, Shenshu Yellow, Yamaguchi Koudaka, Texas Early Grano 502, experimental hybrid XPH 3371 F1 (Asgrow), hybrids 70723 (B1717BxB2923B) and 70719 (B2371CxB2923B), inbred lines B2355B, B2923B, MSU2935B, MSU5718B, MSU8155B.

Flower buds at a mature stage but before dehiscence were taken from above-referenced greenhouse grown donor plants and were surface sterilized by dipping for 10 min in 16.6 g/l dichloroisocyanuric acid Na_2 salt with the addition of a few drops of Tween 20. Subsequent to this treatment the flowers were washed 3 times in sterilized water.

In embodiment 1 (of the claimed process), flower buds were cultured in Petri dishes 100 mm in diameter (30 per dish) on induction media described later. The flowers were on induction media for 6 days (where not said otherwise). After the induction period, flowers were subcultured on Petri dishes containing differentiation media, as described in the following paragraphs.

The embodiment 2 (of the claimed process) differed from embodiment 1 in that the flowers were cultured on induction media and were extracted (perianth removed) before transfer to differentiation media ovaries.

Petri dishes were sealed with Parafilm™ (American National Can, Greenwich, CT, USA) to prevent evaporation and exposed to a 16/8 hours photoperiod at 21-23 ^{o}C and illumination of approximately 80 $\mu mol\ m^{-2}s^{-1}$.

Media were prepared according to established protocols for plant tissue culture work, as described for example in RLM Pierik (1987) *In vitro* culture of higher plants. Martinus Nijhoff Publishers, Dordrecht, Boston, Lancaster, p.344.

The basal medium consisted of BDS macro, micro elements and vitamins (Dunstan and Short 1977, commercially available at Duchefa Biochemie BV Haarlem, Netherlands), 500 mg/l inositol, 200 mg/l proline, 100g/l sucrose (where not said otherwise), the pH was adjusted to 6.0 before autoclaving. Other media components for all exemplified treatments are listed in Tables 11 A and 11B.

Flower buds grown on induction media opened after the first few days, the ovaries enlarged significantly. Flowers and ovaries cultured on induction media and transferred to differentiation media formed the first visually perceptible structures after 3 weeks in culture. The formed structures were particularly notable in the second process embodiment (Protocol 2), where the perianth did not obstruct the view. At this stage, regenerated structures had a globular embryogenic appearance and were completely white (Fig. 1). Further development was evident within one week. The first visual shoot organogenesis appeared on globular structures in the following week (Fig. 2). A part of the shoot structures already elongated in the next 2 weeks on the differentiation medium, individual shoots being approximately 2 cm long (Fig. 3). Such shoots were divided and subcultured on the elongation media, on which they elongated and produced normal plantlets. A proportion of these organogenic structures remained as nodular bumps. When such clusters were subcultured on hormone free media, elongation of shoots occurred, although the organogenic potential was preserved for at least 3 subcultures. The most suitable medium for elongation of shoots was basal medium (or half strength basal medium) excluding phytohormones, with the concentration of sucrose or glucose lowered (20-70g/l, recommendable concentration 30g/l). Shoots gradually developed a green coloration, started to elongate and formed roots in the same way as shoots micropropagated using other standard methods. For accelerated root growth, the media could additionally contain auxins such as 0.5-1.0 mg/l indolebutyric acid (IBA).

The number of individual shoots per flower or ovary was difficult to determine because smaller compact organogenic structures were present in addition to elongated shoots. The average cluster at the end of subculture was composed of 5-10 elongated shoots, the rest being compact organogenic tissue.

The results presented in Tables 1-10 represent the number of flowers (first process embodiment »Protocol 1«) or ovaries (Protocol 2) producing organogenic multiple shoot structures. Statistical differences determined separately for each genotype were tested with ANOVA followed by Duncan's multiple range test (p=0.05). Identical letters following values indicate no significant difference.

Example 1:

Effects of gelling agents

Media solidified using gellan gum (I1/D1), agar/gellan gum mixture (I2/D2) or agar (Difco-Bacto™, Difco Laboratories, Detroit, MI USA) (I3/D3), were tested using Protocol 1 and Protocol 2. Flower buds were transferred from induction media to differentiation media after 6 days. Results are presented in Table 1.

Table 1:

	Flowers		Ovaries	
Medium	No. of inoculated flowers	Organogenic flowers (%)	No. of excised ovaries	Organogenic ovaries (%)
(I1/D1)	419	42.0 b	797	35.9 c
(I2/D2)	420	24.3 ab	814	24.0 b
(I3/D3)	388	15.7 a	872	10.7 a

The highest regeneration percentage of shoot differentiation occurred on media solidified with gellan-gum in both processes but more shoots exhibited a hyperhydrated appearance on gellan-gum solidified media than on agar or agar/gellan-gum mixture.

Example 2:

Duration of induction treatment

The varying duration of the induction stage was studied using the embodiment 2, I1/D1 media. The duration of the induction stage (3, 6 or 12 days) had a significant effect on regeneration. Results are presented in Table 2.

Table 2:

Duration of induction treatment	No. of inoculated ovaries	Organogenic ovaries (%)	Occurrence of hyperhydration (%)
3 days	300	43.0 b	41.1
6 days	300	61.0 c	29.8
12 days	319	17.6 a	62.5

The duration of the induction stage had a significant effect on regeneration. The highest shoot regeneration was observed on ovaries that were placed on differentiation media after 6 days. Shorter or longer induction treatment resulted in decreased regeneration. At 6 days, the lowest hyperhydration also occurred.

Example 3

Effect of source of carbohydrates

To evaluate the optimal source of carbohydrates in induction and differentiation media, 50 g/l (I4/D4) sucrose was compared to an equimolar concentration of glucose (26.3 g/l - I6/D6) and maltose (52.6g/l - I7/D7). In this study, 3 genotypes and Protocol 2 were used. The results are presented in Table 3.

Table 3:

Medium	Genotype	No. of inoculated ovaries	Organogenic ovaries (%)	Occurrence of hyperhydration (%)
I4/D4	70719	177	19.2 a	16.2
I6/D6	70719	174	23.0 a	12.5
I7/D7	70719	180	16.1 a	13.8
I4/D4	Belokranjka	154	18.2 a	25.0
I6/D6	Belokranjka	119	10.1 a	16.7
I7/D7	Belokranjka	138	8.0 a	9.1
I4/D4	Ptujska rdeča	173	15.6 a	11.1
I6/D6	Ptujska rdeča	155	25.2 a	10.3
I7/D7	Ptujska rdeča	155	21.3 a	15.2

Organogenic structures occurred in all media and with all genotypes. There were no statistically significant differences among treatments, and a small difference in the occurrence of hyperhydration.

Example 4:

Effect of sucrose concentration in media

The influence of sucrose concentration in induction and differentiation media was studied using 3 genotypes and embodiment 2. Three different concentrations of sucrose used were: 100 g/l (I1/D1), 50 g/l (I4/D4) and 25 g/l (I5/D5). The results are presented in Table 4.

Table 4:

Medium	Genotype	No. of inoculated ovaries	Organogenic ovaries (%)	Occurrence of hyperhydration (%)
(I1/D1)	2923B	167	18.0 a	20.0
(I4/D4)	2923B	294	22.8 a	11.9
(I1/D1)	Belokranjka	539	21.3 a	13.9
(I4/D4)	Belokranjka	538	26.2 a	3.5
(I5/D5)	Belokranjka	541	25.7 a	5.0
(I1/D1)	MSU5718B	446	6.7 a	25.0
(I4/D4)	MSU5718B	451	17.7 b	11.2
(I5/D5)	MSU5718B	440	11.1 a	24.5

Organogenic structures occurred in all media and with all genotypes. Medium supplemented with 50 g/l sucrose was significantly superior to 100 g/l and 25 g/l with genotype MSU5718B. In the other two genotypes, a lower content of sucrose resulted in a lower occurrence of hyperhydration.

Example 5:

Effect of auxin composition in induction media

The effect of auxin composition in induction media on 4 genotypes using the Protocol 2 was studied. Induction media contained 2 mg/l 2,4 D (I4), 1 mg/l picloram (I8), 2 mg/l picloram (I9) or 5 mg/l NAA (I10). The differentiation media were D1 and D4. The results are presented in Table 5.

Table 5:

Medium	Genotype	No. of inoculated ovaries	Organogenic ovaries (%)	Occurrence of hyperhydration (%)
I10/D1	MSU8155B	316	0	0
I10/D1	B2923B	333	0	0
I4/D4	Belokranjka	845	35.6 b	3.6
I8/D4	Belokranjka	540	11.7 a	13.5
I9/D4	Belokranjka	538	14.9 a	23.7
I4/D4	MSU5718B	362	26.2 b	11.0
I8/D4	MSU5718B	504	6.7 a	16.2
I9/D4	MSU5718B	476	6.9 a	27.3

Organogenic structures occurred on three of four media. Medium I4/D4 was significantly superior to the other two combinations with both genotypes. The occurrence of hyperhydration was the lowest on medium I4/D4.

Example 6:

Effect of cytokinin composition in induction media

The effect of cytokinin composition in the induction media on 2 genotypes using Protocol 2 was studied. Induction media contained 2 mg/l BAP (I4), cytokinin omitted (I11), 1 mg/l TDZ (I12) and 2 mg/l 2ip (I13). The differentiation medium was D4. The results are presented in Table 6.

Table 6:

Medium	Genotype	No. of inoculated ovaries	Organogenic ovaries (%)	Occurrence of hyperhydration (%)
I4/D4	Belokranjka	845	35.6 c	3.6
I11/D4	Belokranjka	540	5.6 a	8.4
I12/D4	Belokranjka	532	43.2 c	0.4
I13/D4	Belokranjka	538	16.5 b	4.5
I4/D4	MSU5718B	362	26.2 c	11.0
I11/D4	MSU5718B	484	8.5 a	12.2
I12/D4	MSU5718B	482	19.3 bc	12.4
I13/D4	MSU5718B	482	12.2 ab	27.1

The highest regeneration was obtained on medium I12, the difference was statistically significant compared to I11 and I13 (first genotype), with the second genotype the highest regeneration was obtained on I4 medium. The percentage of hyperhydrated shoots was also low on I12 medium. Omitting cytokinin in the induction media (I11) greatly reduced regeneration.

Example 7:

Effect of cytokinin composition of differentiation media

The effect of cytokinin composition in the differentiation media on 4 genotypes using Protocol 2 was studied. Effects of cytokinins were studied at 2 sucrose concentrations. Differentiation media contained 2 mg/l TDZ (D1) or 5 mg/l BAP (D10 and D11), induction media were I1 or I4. The results are presented in Table 7.

Table 7:

Medium	Genotype	No. of inoculated ovaries	Organogenic ovaries (%)	Occurrence of hyperhydration (%)
I1/D1	Belokranjka	718	39.4 b	8.8
I1 1/D10	Belokranjka	330	4.5 a	36.7
I4/D4	Belokranjka	845	35,6 c	3.6
I4/D8	Belokranjka	537	17.3 a	9.7
I4/D9	Belokranjka	541	27.0 b	2.7
I1/D1	70723	97	27.8 a	33.4
I4/D4	70723	98	54.1 a	9.4
I1/D10	70723	97	28.9 a	23.2
I4/D11	70723	97	43.3 a	5.9
I4/D4	MSU5718B	362	26.2 a	11.0
I4/D8	MSU5718B	425	26.4 a	9.8
I4/D9	MSU5718B	423	21.0 a	9.0
I1/D1	B2923B	164	12.8 a	28.6
I4/D4	B2923B	167	43.1 b	22.3
I1/D10	B2923B	166	18.7 a	32.3
I4/D11	B2923B	166	27.1 a	20.0

Organogenic structures occurred with all studied media and genotypes.

Basal callus structures grew on all media types and with all genotypes. The low sucrose level increased regeneration with all genotypes and lowered the hyperhydration.

Example 8:

Effect of light

Formation of organogenic structures in light or in darkness was studied using Protocol 2. Two genotypes and two media (I4 and D4) were studied. The results are presented in Table 8.

Table 8:

Treatment (Day or night)	Genotype	No. of inoculated ovaries	Organogenic ovaries (%)	Occurrence of hyperhydration (%)
Day	Belokranjka	539	34.3 b	2.2
Night	Belokranjka	550	18.7 a	9.7
Day	MSU5718B	433	21.2 a	15.2
Night	MSU5718B	425	17.6 a	4.7

Organogenic structures occurred with both studied media and genotypes, regeneration in daylight was superior to regeneration in darkness.

Example 9:

Effect of composition of micro and macro elements

Various contents of macro and micro elements in induction and differentiation media were studied in two genotypes using Protocol 2. Induction and differentiation media included BDS medium (I4/D4), B5 medium (I14/D12) or MS medium (I15/D13). The results are presented in Table 9.

Table 9:

Medium	Genotype	No. of inoculated ovaries	Organogenic ovaries (%)	Occurrence of hyperhydration (%)
(I4/D4)	Belokranjka	486	39.5 b	4.9
(I14/D12)	Belokranjka	512	23.4 a	5.0
(I15/D13)	Belokranjka	534	30.7 ab	11.3
(I4/D4)	MSU5718B	459	21.8 a	14.5
(I14/D12)	MSU5718B	459	19.2 a	7.4
(I15/D13)	MSU5718B	442	24.9 a	11.4

Organogenic structures occurred on all studied media and with both genotypes. The lowest hyperhydration occurred on media I14/D12.

Example 10:

Determination of genotypic effect

Influence of genotype was studied using Protocols 1 and 2 and media I1/D1. The occurrence of organogenic structures for 12 genotypes of onion are presented in Table 10.

Table 10 (Protocol 1: first embodiment of the claimed process):

Genotype	No. of inoculated flowers	Organo-genic flowers (%)	Ovaries producing basal callus (%)	No. of shoots produced on basal callus	Occurrence of hyper-hydration (%)
Belokranjka	712	45.4 ef	16.3	6.5	2.3
Stuttgarter Riesen	897	20.1 abc	18.6	6.7	5.2
Timor	399	10.8 ab	13.5	5.3	5.5
Shenshu Yellow	326	17.2 abc	22.4	5.5	10.1
Yamaguchi Koudaka	577	31.2 cde	9.5	2.8	0.5
XPH3371 F1 (Asgrow)	368	25.5 bcd	13.3	7.9	2.0
Texas Early Grano 502	309	15.9 abc	27.5	9.1	5.2
Inb. line B2355B	477	19.5 abc	26.6	9.2	3.3
Inb. line B2923B	1139	39.2 def	24.1	5.4	20.8
Inb. line MSU2935B	446	57.2 f	8.8	2.5	13.2
Inb. line MSU5718B	529	44.0 def	24.6	4.9	25.3
Inb. line MSU8155B	748	3.6 a	2.5	1.1	10.0

Table 11 (Protocol 2: second emb diment of the claimed process):

Genotype	No. of inoculated ovaries	Organogenic ovaries (%)	Occurrence of hyperhydration (%)
Belokranjka	718	39.4 d	8.8
Stuttgarter Riesen	947	14.6 ab	6.1
Timor	319	29.2 bcd	30.1
Shenshu Yellow	419	25.8 bcd	15.7
Yamaguchi Koudaka	584	28.8 bcd	14.6
XPH3371 F1 (Asgrow)	357	15.9 abc	18.4
Texas Early Grano 502	318	32.1 cd	3.4
Inb. line B2355B	467	37.0 d	41.3
Inb. line B2923B	1339	14.9 ab	22.4
Inb. line MSU2935B	627	25.5 bcd	21.6
Inb. line MSU5718B	616	57.9 e	13.2
Inb. line MSU8155B	955	4.1 a	23.1

All tested varieties produced multiple organogenic structures using both embodiments of the claimed process. When flowers were cultured on induction and differentiation media (Protocol 1), a basal callus developed on the bases of some flowers. Occasionally, adventitious shoot regeneration occurred on such calli, and were scored separately from those produced *via* direct organogenesis. When the ovaries were extracted (Protocol 2) no callus was formed. The induction efficiency differed and differences among cultivars were statistically significant in both embodiments of the claimed process. The highest regeneration rate was 57.9% (Protocol 2) and 45.4% (Protocol 1). It should be noted that the line MSU8155B, exhibiting the lowest regeneration rate using both embodiments, flowered 3 weeks later than the others, so in addition to genotype, an environmental

effect (higher temperature in greenhouse) could also have had an impact on the lower regeneration. One half of the varieties responded with similar induction frequencies to both embodiments of the claimed process, while others differed, some responding better using Protocol 1 and some using Protocol 2. The appearance of hyperhydrated shoots also differed among cultivars, and was lower on flowers (Protocol 1) than on ovaries (Protocol 2).

TABLE 11A

induction media

	I1	I2	I3	I4	I5	I6	I7	I8	I9	I10	I11	I12	I13	I14	I15
Macro and micro elements															
BDS	+	+	+	+	+	+	+	+	+	+	+	+	+		
B5														+	
MS															+
Vitamins (mg/l)															
m-Inositol	500	500	500	500	500	500	500	500	500	500	500	500	500	500	500
Thiamine	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Pyridoxine	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Niacin	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
L-Proline	200	200	200	200	200	200	200	200	200	200	200	200	200	200	200
Carbohydrates(g/l)															
Sucrose	100	100	100	50	25			50	50	100	50	50	50	50	50
Glucose						26.3									
Maltose							52.6								
Phytohormones (mg/l)															
2,4-D	2	2	2	2	2	2	2				2	2	2	2	2
NAA										5					
Picloram								1	2						
TDZ												1			
BAP	2	2	2	2	2	2	2	2	2	2				2	2
2ip*													2		
Gelling agents (g/l)															
Agar			7												
Agar Gellan-gum		3.5 1													
Gellan-gum	2			2	2	2	2	2	2	2	2	2	2	2	2

* filter sterilised and added to media after autoclaving

TABLE 11B

differentiation media

	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	
Macro and micro elements														
BDS	+	+	+	+	+	+	+	+	+	+	+	+		
B5													+	
MS														+
Vitamins (mg/l)														
m-Inositol	500	500	500	500	500	500	500	500	500	500	500	500	500	
Thiamine	10	10	10	10	10	10	10	10	10	10	10	10	10	
Piridoksine	1	1	1	1	1	1	1	1	1	1	1	1	1	
Niacin	1	1	1	1	1	1	1	1	1	1	1	1	1	
L-Prolin	200	200	200	200	200	200	200	200	200	200	200	200	200	
Carbohydrate (g/l)														
Sucrose	100	100	100	50	25			50	50	100	50	50	50	
Glucose						26.3								
Maltose							52.6							
Phytohormones (mg/l)														
TDZ	2	2	2	2	2	2	2	0.5	1			2	2	
BAP										5	5			
Gelling agents (g/l)														
Agar			7											
Agar Gellan-gum		3.5 1												
Gellan-gum	2			2	2	2	2	2	2	2	2	2	2	

What is claimed is:

1. A process for induction of direct *in vitro* organogenesis in onion, characterized in that it comprises the steps of:

(i) inoculation of flower buds at a mature stage but before dehiscence on induction media, which contain adequate concentrations of phytohormones, growth regulators and gelling agents for initiation of direct somatic organogenesis, the growth regulators at this stage including an effective mixture of auxins and cytokinins,, and the media containing macro and micro elements, vitamins, inositol, proline, carbohydrates and gelling agents;

(ii) after an appropriate induction time transferring the explants from induction to differentiation media which contain adequate concentrations of cytokinin, macro and micro elements, vitamins, inositol, proline, sucrose and gelling agents till direct organogenesis occurs;

(iii) optional removal of perianth before transfer from induction to differentiation media and culture of extracted ovaries, followed by treatment described in (i) and (ii) leading to induction of direct somatic organogenesis;

(iv) separation of globular embryogenic structures developed as described in (ii) and (iii) followed by transfer of elongated shoots to media for root development or maintenance of compact organogenic structures;

(v) acclimatization of previously (iv) obtained rooted shoots.

2. Process according to claim 1 wherein the said induction media contain media solidifiers gellan-gum, agar or a mixture of agar and gellan-gum.

3. A process according to claim 1 wherein the said differentiation media contain media solidifiers gellan-gum, agar, or a mixture of agar and gellan-gum.

4. A process according to claim 1 wherein the growth time on induction media is from 3 to 12 days.
5. A process according to claim 1 wherein the induction and differentiation media contain sucrose, glucose or maltose as a source of carbohydrates.
6. A process according to claim 1 wherein the said induction and/or differentiation media contain 25-100g/l of sucrose.
7. A process according to claim 1 wherein the said induction media contain cytokinin and 2,4-dichlorophenoxyacetic acid or picloram. as a sources of auxins.
8. A process according to claim 1 wherein the said induction media contain auxin or auxin and 6-benzylaminopurine, thidiazuron or isopentenyladenin (2ip) as sources of cytokinins.
9. A process according to claim 1 wherein the said differentiation media contain thidiazuron or 6-benzylaminopurine as sources of cytokinins.
10. A process as claimed in claim 1 wherein the said induction is being established in light or in darkness.
11. A process as claimed in claim 1 wherein the said differentiation is being established in light or in darkness.
12. A process as claimed in claim 1 wherein the said composition of macro and micro elements in induction media corresponds to BDS media, B5 media or MS media.
13. A process as claimed in claim 1 wherein the said composition of macro and micro elements in differentiation media corresponds to BDS media, B5 media or MS media.

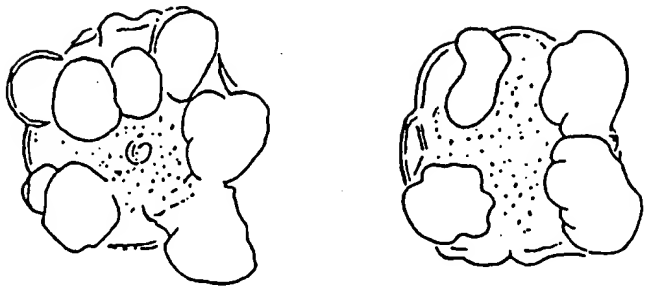


FIG. 1

FIG. 2

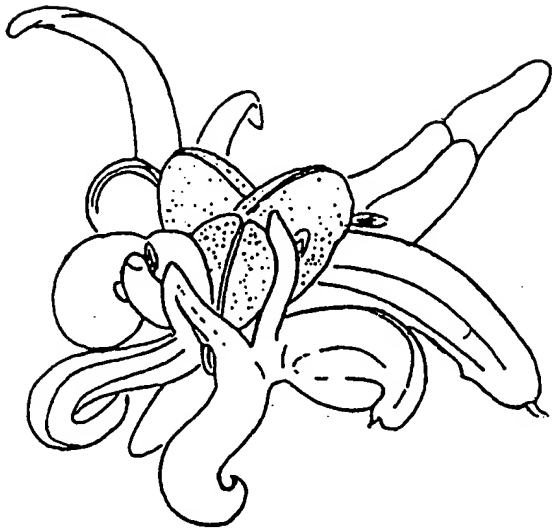


FIG. 3

INTERNATIONAL SEARCH REPORT

Intern. ..nal Application No
PCT/SI 99/00022

A. CLASSIFICATI N OF SUBJECT MATTER
IPC 7 A01H4/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	COHAT, J.: "Obtention chez l'échalote (Allium cepa L var aggregantum) de plantes haploïdes gynogénétiques par culture in vitro de boutons floraux" AGRONOMIE, vol. 14, 1994, pages 299-304, XP000879486 page 300, right-hand column, paragraph 2 - paragraph 3 page 303, left-hand column, last paragraph -right-hand column, paragraph 1	1-13
Y	EP 0 814 166 A (DEKALB GENETICS CORP) 29 December 1997 (1997-12-29) page 10, line 33 - line 34 page 12; example 501; table 1	1-13

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 March 2000	13/03/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Fonts Cavestany, A

2

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter. .onal Application No
PCT/SI 99/00022

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US AN-1996:509121, 1996 JAKSE, M. ET AL.: "Effect of media components on the gynogenic regeneration of onion (Allium cepa L.) cultivars and analysis of regenerants." XP002131953 cited in the application & Plant Cell Reports Vol. 15, No. 12, pp. 934-938 abstract	1-13
A	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US AN-1995:172234, 1995 BOHANEC, B.: "Studies of gynogenesis in onion (Allium cepa L.): induction procedures and genetic analysis of regenerants." XP002131954 & Plant Science, Vol. 104, No. 2, pp.215-224. abstract	1-13
A	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US AN - 1993:9054, 1992 CAMPION, B. ET AL.: "Advances in haploid plant induction in onion (Allium cepa L.) through in vitro gynogenesis" XP002131955 &Plant Science, 1992 Vol 86 No 1 pp 97-104 abstract	1
A	DATABASE CAB 'Online! CAB INTERNATIONAL, WALLINGFORD, OXON, GB AN-97:83769, 1997 JEONG HAEBOONG ET AL.: "Plant redifferentiation and in vitro multiplication of onion by shoot primodium culture" XP002131956 & Journal of the Korean Society for Horticultural Science, Vol. 38, No. 2, pp. 123-128 abstract	1

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern. nal Application No
PCT/SI 99/00022

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0814166 A	29-12-1997	AT 160174 T	15-11-1997
		AU 644097 B	02-12-1993
		AU 6290390 A	11-03-1991
		CA 2064761 A	10-02-1991
		DE 69031711 D	18-12-1997
		DE 69031711 T	09-04-1998
		EP 0485506 A	20-05-1992
		ES 2110417 T	16-02-1998
		HU 60781 A	28-10-1992
		JP 5501352 T	18-03-1993
		WO 9102071 A	21-02-1991
		US 5489520 A	06-02-1996
		US 5969213 A	19-10-1999
		US 5874265 A	23-02-1999
		US 5919675 A	06-07-1999
		US 5550318 A	27-08-1996